Supporting Information

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SI Materials and Methods

Patients. Ipilimumab is a fully human monoclonal Ig (IgG1) specific for human CTLA-4 (CD152). The antibody was given at a dose of 3 mg/kg each time, with a 3-week interval between doses. Blood was collected pre-therapy before the first dose, at week 3 before the second dose (post-therapy week 3), and at week 7 before surgery (post-therapy week 7). Additional blood was collected on an IRB-approved lab protocol to obtain sufficient cells for immunological analyses. Tissues were collected from surgical samples. All patients were monitored for safety. This trial is an ongoing clinical trial, and to date six of six patients have completed all treatments, including surgical removal of their bladders. The results reported here reflect data obtained from all six treated patients. One patient (patient 2) did not have blood collected at week 7 secondary to a delay in the surgery from week 7 to week 11 because of a non-drug-related preoperative cardiac intervention. Tissues from similarly staged bladder cancer patients undergoing radical cystectomy ($n = 10$) who did not participate in the anti-CTLA-4 clinical trial but were consented onto a separate IRB-approved sample acquisition laboratory protocol were used as untreated control samples. Blood from untreated bladder cancer patients and from the pre-therapy samples of treated patients served as control samples for data obtained from post-therapy blood samples of anti-CTLA-4-treated patients.

Blood and Tissue Processing. Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation using Lymphocyte Separation Medium (Mediatech) and Leucosep tubes (Greiner Bio-one). Cells recovered from the gradient interface were washed twice in RPMI medium 1640 (Mediatech), counted, and used in multiple assays. Fine needle aspirations of bladder cancer tissues were obtained from radical cystectomy surgical specimens with an 18-gauge needle that was passed through tumor tissues multiple times to obtain a liquid volume of ≈5 ml of material per sample. The specimens were then washed twice with cold PBS supplemented with 2% BSA and 2 mM EDTA before performing cell surface and intracellular staining for *ex vivo* flow cytometric analyses. Ureter tissues from radical cystectomy surgical cases of untreated patients that were confirmed by pathology review (P.T.) to be disease-free were used as nonmalignant controls. Cells from ureters were obtained from Seward Stomacher 80 Lab Blender (Cole-Palmer Instrument Company) after 6 min of homogenization in RPMI medium 1640. Tissues from bladder and ureter surgical samples were also stored in RNA later (Ambion) at −80°C for PCR analyses. Data presented here represent 10 untreated bladder tumors, 4 ureter (non-malignant) specimens from untreated patients, and 6 anti-CTLA-4-treated bladder tumor samples.

Flow Cytometry. Antibodies used for flow cytometry consisted of: CD3-FITC and CD4-PerCP-Cy5.5 (BD PharMingen), FOXP3-PE (eBiosciences, clone PHC101), CD25-APC, and ICOS-biotilynated (eBiosciences) conjugated with streptavidin-APC-Cy7 (BD Biosciences). Intracellular staining for FOXP3 was conducted as per the manufacturer's guidelines. Triplicate samples were analyzed by using the FACSCanto II (Becton Dickinson). Data were analyzed by using BD FACSDiva software. Gates were set according to appropriate isotype controls. Events collected were: 1,000,000 events for blood samples, approximately 100,000 to 200,000 events per tumor sample, and approximately 30,000 to 50,000 events per ureter samples.

Cell Culture and Stimulation. CD4 T cells from peripheral blood mononuclear cells (PBMCs) were obtained by positive immunoselection using microbeads (Miltenyi Biotec), and 100,000 cells per well in triplicate wells were cultured in freshly made RPMI medium 1640 with 2 mM L-glutamine (Mediatech), 5 mM Hepes, 0.05 mM nonessential amino acids, 0.5 mM sodium pyruvate, 100 U/ml penicillin/ug/ml streptomycin (all from Invitrogen), and 5% human AB serum (Gemini Bio-Products) in 96-well U-bottom plates (Costar) with 1 μg/ml plate-bound anti-CD3 antibody (BD Biosciences) and 500 U/ml IL-2 (Chiron) for 3 days before collecting supernatant for cytokine analysis and addition of 1 uCi ^{3}H-thymidine (Amersham Biosciences) for 18 h, then assessing proliferation with a MicroBeta TriLux scintillation counter (PerkinElmer). Uncultured fresh CD4 T cells were also sorted *ex vivo* by FACS (Aria; BD Biosciences) according to their ICOS phenotype. Sorted ICOS^{hi} and ICOS^{low} cells were plated at 50,000 cells per well in triplicate wells with 1 μg/ml anti-CD3 antibody and 500 U/ml IL-2 for 3 days before supernatant being collected for cytokine analysis. Cytokine analyses were performed by using the human Th1/Th2 7-plex Ultra-Sensitive Kit (Meso Scale Discovery], which has a limit of detection of 0.5 pg/ml for both IFN-γ and IL-10. Suppression assays were performed by sorting PBMCs for CD4^{+}CD25^{-} and autologous CD4^{+}CD25^{hi} cells, with each plated alone at 5,000 cells per well or together at a 1:1 ratio in triplicate wells for 6 days with 5 μg/ml anti-CD3 antibody, and then adding 1 uCi ^{3}H-thymidine for 18 h before assessing proliferation with a scintillation counter.

Real-Time PCR. Total RNA samples from tissues and CD4 T cells were isolated by RNeasy kit (Qiagen). Reverse transcription was performed by using SuperScript™ III Reverse Transcriptase kit (Invitrogen). Real-time quantitative PCR was performed with the 7500 Fast Real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Samples were used as templates in reactions to obtain the threshold cycle (Ct) that were normalized with the Ct of GAPDH or CD3-ε from the same sample (ΔCt). To compare the relative levels of gene expression in different tissues, $\Delta\Delta$Ct values were calculated, with the ΔCt values associated with the lowest expression levels as 1. Fold induction was calculated by using $2^{\Delta\Delta Ct}$. CD3-ε, IFN-γ, T-bet, and FOXP3 probes were synthesized by Taqman Gene Expression Assay (Applied Biosystems). Other primers used were synthesized by Integrated DNA Technologies and were used with SYBR Green PCR Master Mix System (Applied Biosystems). Primers used for PCR include GAPDH sense: TGCACCACCAACTGCTTAGC and anti-sense: GGCATGGACTGTGGTCATGAG, IL-2 sense: AAGTTTTTACATACCCAAGAAGG and antisense: AAGTGAAAGTTTTTGCTTTGAGC, IL-10 sense: TGGGGGAGAACC TGAAGAC and antisense: ACAGGGAAGAAATCGATGACA, GATA-3 sense: GCTTCGGATGCAAGTCCA and antisense: GCCCCACAGTTCACACACT, and IL-4 sense: CACCGAGTTGACCGTAACAG and antisense: GCCCTGCAGAAGGTTTCC.

ELISpot Assays. To serve as antigen-presenting cells (APCs), peripheral blood mononuclear cells (PBMCs) were depleted of CD8^{+} and CD4^{+} T cells and pulsed with a 10 μM peptide pool consisting of synthesized (Multiple Peptide Systems) 20-mer overlapping peptides (amino acids 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 71–90, 81–100, 91–110, 101–120, 111–130, 119–143, 131–150, 139–160, 151–170, and 161–180) encompassing the full-length NY-ESO-1 antigen. Pulsed APCs were added to the plates containing CD4^{+}ICOS^{hi} T cells and supplemented

with RPMI media 1640. IL-2 (10 U/ml, Roche Molecular Biochemicals) and IL-7 (20 ng/ml, R&D Systems) were added to culture wells, and cells were harvested after 3 weeks for testing. Sensitized $CD4^+ICOS^{hi}$ T cells were then tested with target APCs without peptide or pulsed with peptide in ELISPOT assays as previously described (30). Nitrocellulose plates (MultiScreen-HA; Millipore) were coated with IFN-γ mAb (4 μg/ml, 1-D1K; Mabtech) and incubated overnight at 4°C. The presensitized $CD4^+ICOS^{hi}$ T cells and target APCs were added to each well and incubated for 20 h. Plates were then washed, IFN-γ mAb (0.2 μg/ml, 7-B6–1-biotin; Mabtech) was added, and the plates were incubated for 2 h at 37°C, washed again, and developed with streptavidin-alkaline phosphatase (1 μg/ml; Roche). Substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma) was added, and plate membranes displaying dark-violet spots representing IFNγ-secreting T cells were counted with CTL Immunospot analyzer and software (Cellular Technologies).

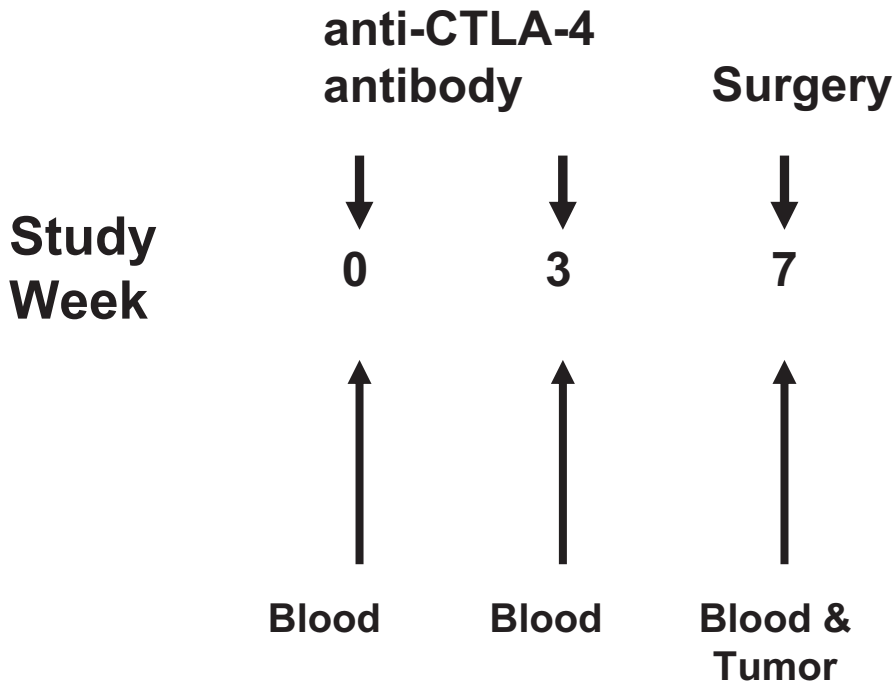

Fig. S1. Clinical trial schema for the administration of anti-CTLA-4 antibody and collection of blood and surgical tissue samples for analyses.

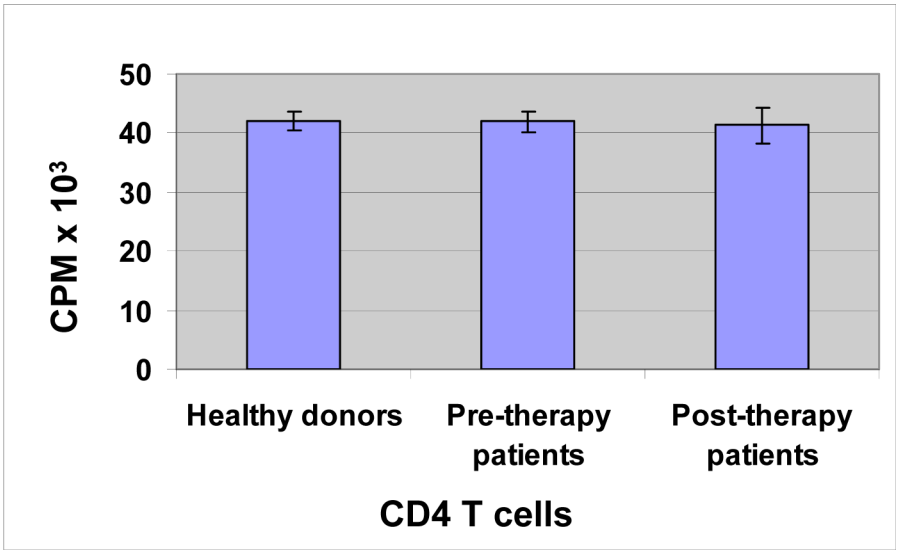

Fig. S2. Proliferation of CD4 T cells. CD4 T cells proliferated equally well from healthy donors, patients with untreated bladder cancer (pretherapy patients), and patients treated with anti-CTLA-4 therapy (post-therapy patients).

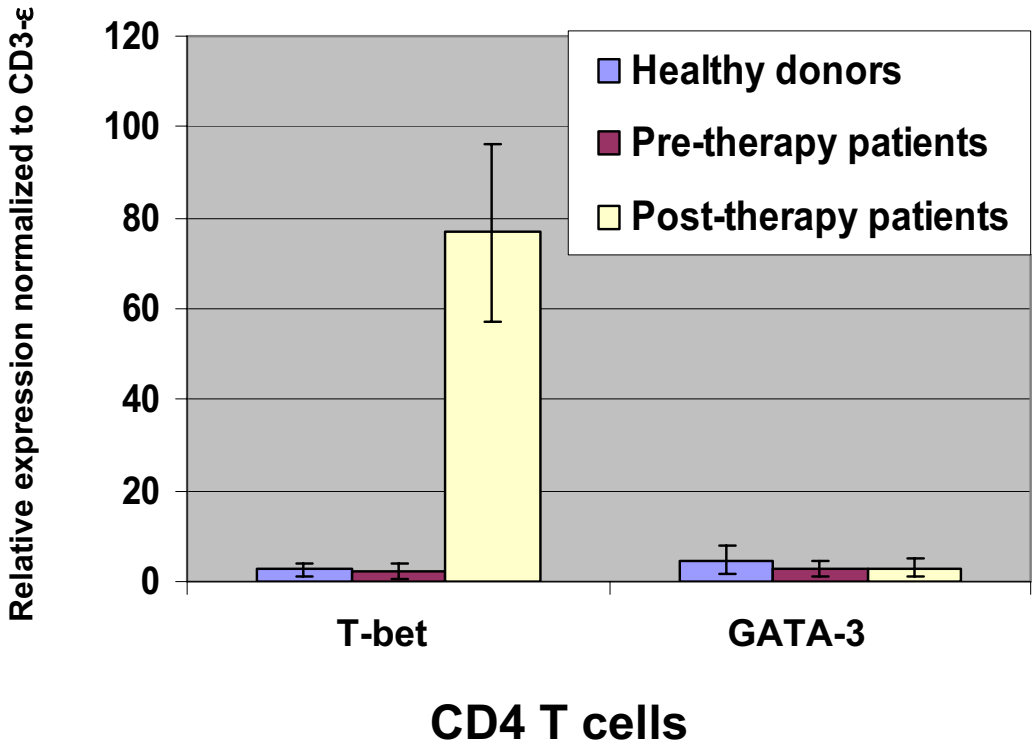

Fig. S3. Fold induction of T-bet and GATA-3 mRNA levels relative to CD3-ε mRNA expression in CD4 T cells from peripheral blood.

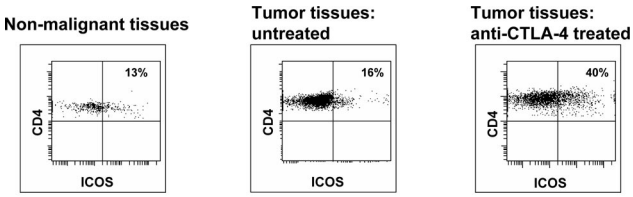

Fig. S4. FOXP3 expression by tumor-infiltrating CD4 T cells was decreased after anti-CTLA-4 treatment. Data shown are from representative samples. (*Left*) Nonmalignant urothelial tissues from an untreated bladder cancer patient. (*Center*) Urothelial carcinoma tissues from an untreated bladder cancer patient. (*Right*) Urothelial carcinoma tissues from a bladder cancer patient treated with anti-CTLA-4 antibody.

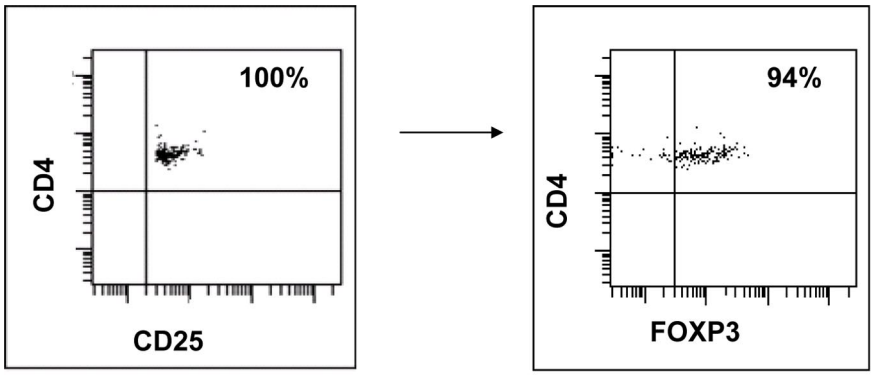

Fig. S5. $CD4^+FOXP3^+$ T cells function as regulatory T cells. (*A*) Representative flow cytometric data demonstrating that gated $CD25^{hi}$ cells (labeled as $CD4^+CD25^+$ T cells) (*Left*) from one patient (patient 1) comprised a large population of $FOXP3^+$ cells (*Right*). (*B*) Healthy donors, untreated bladder cancer patients (pretherapy patients), and bladder cancer patients treated with anti-CTLA-4 therapy (post-therapy patients) have $CD4^+CD25^-$ cells that were proliferative when cultured alone, but autologous $CD4^+CD25^+$ T cells proliferated minimally in culture alone and inhibited proliferation of $CD4^+CD25^-$ cells when the two cell populations were cultured together at a 1:1 ratio.

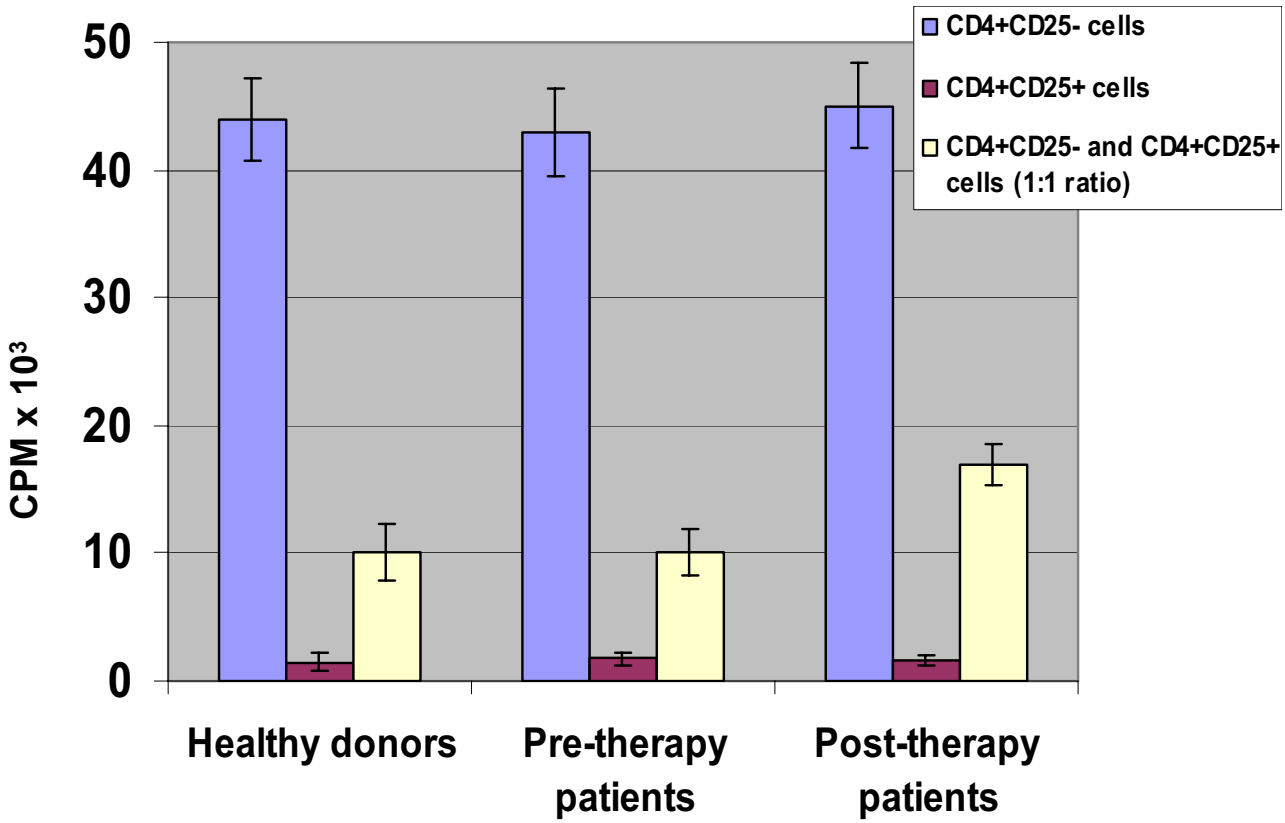

Fig. S5. Continued.

Table S1. Mean ratio calculated from tumor tissues of 10 untreated bladder cancer patients compared to tissues from 6 patients who received anti-CTLA-4 therapy

Tumor tissues	Ratio of $CD4^+ICOS^{hi}$ to $CD4^+FOXP3^+$ T cells
Untreated bladder cancers ($N = 10$)	0.2
Patient #1 post-therapy	5.5
Patient #2 post-therapy	4.3
Patient #3 post-therapy	6.2
Patient #4 post-therapy	3.4
Patient #5 post-therapy	6.4
Patient #6 post-therapy	4.5